PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing: 31 July 1997 (31.07.97)	
International application No.: PCT/GB97/00210	Applicant's or agent's file reference: KP VM/1640
International filing date: 23 January 1997 (23.01.97)	Priority date: 23 January 1996 (23.01.96)
Applicant: KINGSMAN, Alan, John et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

15 July 1997 (15.07.97)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

PRIVETT, Kathryn L
STEVENS, HEWLETT & PERKINS
1 Serjeant's Inn
Fleet Street
London EC4Y 1LL
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day month year)*	27.04.98
Applicant's or agent's file reference KP/VM/1640	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB97/00210	23/01/1997	23/01/1996

Applicant
OXFORD BIOMEDICA (UK) LIMITED et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA
European Patent Office

Authorized officer
Vullo, C

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:
STEVENS, HEWLETT & PERKINS
Attn. PRIVETT, Kathryn L.
1 Serjeants' Inn
Fleet Street
LONDON EC4Y 1LL
UNITED KINGDOM

30.5(IC)

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference KP/VM/1640	Date of mailing (day/month/year) 14/05/1997
International application No. PCT/GB 97/00210	FOR FURTHER ACTION See paragraphs 1 and 4 below
Applicant OXFORD BIOMEDICA PLC et al.	International filing date (day/month/year) 23/01/1997

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicants's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

... months

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk	Authorized officer Zorka Bota

Form PCT/ISA/220 (January 1994)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

[illegible] of the designated/elected Offices see Volume II of the PCT Applicant's

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
KP/VM/1640		
International application No.	International filing date (day/month/year)	(Earliest) Priority Date (day/month/year)
PCT/GB 97/00210	23/01/1997	23/01/1996
Applicant		
OXFORD BIOMEDICA PLC et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [ ] the text is approved as submitted by the applicant.
   - [X] the text has been established by this Authority to read as follows:

   RETROVIRAL VECTOR AND ITS USE IN GENE THERAPY

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

drawings

- [ ] because the applicant failed to suggest a figure.
- [ ] because this figure better characterizes the invention.

Form PCT/ISA/

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
KP/VM/1640		
International application No	International filing date (day/month/year)	Priority date (day/month/year)
PCT/GB97/00210	23/01/1997	23/01/1996
International Patent Classification (IPC) or national classification and IPC		
C12N15/86		
Applicant		
OXFORD BIOMEDICA (UK) LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
	27 05 98
Name and mailing address of the IPEA	Authorized officer
European Patent Office	Olsen I

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB97/00210

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-18 as originally filed

Claims, No.:

1-21 as received on 13/03/1998 with letter of 11/03/1998

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 6-14,17-21
	No:	Claims 1-5,15,16
Inventive step (IS)	Yes:	Claims 6-14,17-21
	No:	Claims 1-5,15,16

2. Citations and explanations

see separate sheet

ITEM V

1. The scope of the claims 1 to 5, 15 and 16 covers known prior art such as the prior art already cited in the present description. Claims 1 to 5, 15 and 16 do thus not fulfil the requirements of Article 33 (2) and/or (3) PCT.

Claim 1 concerns a DNA sequence encoding a replication defective retroviral vector comprising at least one heterologous gene, but no functional env and gag- pol genes. Claims 1 further states that the DNA sequence is suitable for administering to a patient by non-retroviral means and for converting cells in a patient into producer cells producing replication defective retroviral vector particles. The prior art disclosed on page 2-4 of the present description discloses DNA sequences which comprise a heterologous gen and lack the env and gag-pol genes. These sequences would appear to be suitable for the administering to and working in cells of a patient even if not so stated (see for example WO 94/29438, which discloses plasmids encoding the env and gag-pol genes and a plasmid comprising the replication defective retroviral vector, all comprisied in a mammalian cell). Hence, the DNA sequence as claimed per se in claim 1 is anticipated by the prior art. Similarly, sets of sequences as claimed in present claim 2 is also anticipated by the prior art, e.g. WO 94/29438.

The subject-matter of claims 3-5 is also deducible from the cited prior art, e.g. WO 94/29438.

The subject-matter of claims 15 and 16 also covers the prior art, for example as disclosed in or suggested by WO 94/29438, as the producer cells and the DNA sequence or set of sequences of the prior art, as exemplified by WO 94/29438, are also used to manufacture a medicament [illegible] the medicament being the replication defective retroviral vector

2. As the subject-matter of claims 1-5. 15 and 16 covers known prior art. it

would also be obvious in view of the same prior art (Article 33(3) PCT).

3. The subject-matter of claims 6-14 and 17-21 would seem to be novel and to involve an inventive step in view of the prior art, as the treatment of cells in the patient or of fresh cells to be introduced into the patient, reflected in the said claims, does not appear to be disclosed in the prior art.

4. For the assessment of the present claims 17-21 on the question whether they are industrially applicable, it must firstly be clarified whether the methods claimed are in vivo or in vitro methods. In the case of in vitro methods, the methods are industrially applicable without limitations. In the case of in vivo methods, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known com- pound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

CLAIMS:

1. A DNA sequence encoding a replication defective retroviral vector for converting cells in a patient into producer cells capable of producing replication defective retroviral vector particles containing the vector, said retroviral vector comprising at least one heterologous gene, which vector contains neither functional *env* nor functional *gag-pol* genes, the DNA sequence in a form suitable for administering to a patient by non-retroviral means and capable of being taken up by the cells, said DNA sequence for use in treatment.

2. A set of DNA sequences for converting cells in a patient into producer cells capable of producing replication defective retroviral vector particles, the set of sequences comprising the DNA sequence according to claim 1 and DNA sequences encoding packaging components Env and Gag-Pol for production of infective retroviral vector particles by the producer cells, the set of DNA sequences in a form suitable for administering to a patient by non-retroviral means and capable of being taken up by the cells, said set of DNA sequences for use in treatment.

3. DNA sequences according to claim 1 or claim 2, wherein the at least one heterologous gene includes at least one therapeutically active gene.

4. DNA sequences according to any one of claims 1 to 3, for converting cells of the patient which are of a target cell type intended for receiving the therapeutically active gene.

5 DNA sequences according to any one of claims 1 to 4 present in one or more plasmids

6. A producer cell for use in treatment, said producer cell capable of producing a replication defective retroviral vector in an infective

retroviral vector particle, the producer cell comprising a set of DNA sequences encoding the replication defective retroviral vector and the packaging components Env and Gag-Pol, said vector comprising at least one heterologous gene and which vector contains neither function Env nor functional *gag-pol* genes, the producer cell being a fresh cell suitable for introduction into a patient and use in gene therapy.

7. The producer cell according to claim 6, wherein the at least one heterologous gene in the vector includes at least one therapeutically active gene.

8. The producer cell according to claim 7, wherein the cell is of a target cell type intended for receiving the therapeutically active gene.

9. The producer cell according to claim 7, wherein the cell is an immune system cell capable of delivering the vector to target cells intended to receive the therapeutically active gene.

10. The producer cell according to any one of claims 6 to 9, for reimplantation into the patient from which it is derived.

11. An *in vitro* method of making a producer cell capable of producing a replication defective retroviral vector in an infective retroviral particle, said vector comprising at least one therapeutically active gene and which vector contains neither functional *env* nor functional *gag-pol* genes, which method comprises introducing a set of DNA sequences encoding the replication defective retroviral vector and packaging components Env and Gag-Pol into a fresh mammalian cell *in vitro* to give a producer cell suitable for use in gene therapy.

12. The method according to claim 11, wherein the producer cell is of a target cell type intended for receiving the therapeutically active gene.

13. The method according to claim 11, wherein the producer cell is an immune system cell capable of delivering the vector to target cells intended to receive the therapeutically active gene.

14. The method according to any one of claims 11 to 13, wherein the fresh cell is from a patient to be treated by gene therapy.

15. Use of a producer cell according to any one of claims 6 to 10, in the manufacture of a medicament for use in gene therapy.

16. Use of a DNA sequence or set of DNA sequences according to any one of claims 1 to 5, in the manufacture of a medicament for use in gene therapy.

17. A method of making a producer cell *in vivo* in a patient, which producer cell is capable of producing a replication defective retroviral vector in an infective retroviral particle, said vector comprising at least one therapeutically active gene and which vector contains neither functional *env* nor functional *gag-pol* genes, which method comprises introducing a set of DNA sequences encoding the replication defective retroviral vector and packaging components Env and Gag-Pol into at least one cell of the patient *in vivo* to give a producer cell.

18. The method according to claim 17, wherein the producer cell is of a target cell type intended for receiving the therapeutically active gene.

19 The method according to claim 17, wherein the producer cell is an immune system cell capable of delivering the vector to target cells intended to receive the therapeutically active gene.

20. A method of performing gene therapy on a patient, which method comprises introducing into the patient a producer cell according to any one of claims 6 to 10.

21. A method of performing gene therapy on a patient, which method comprises introducing into the patient by non-retroviral means a set of DNA sequences according to any one of claims 2 to 5

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/86, 15/87, A61K 48/00

A1

(11) International Publication Number: **WO 97/27310**

(43) International Publication Date: 31 July 1997 (31.07.97)

(21) International Application Number: PCT/GB97/00210

(22) International Filing Date: 23 January 1997 (23.01.97)

(30) Priority Data:

9601336.2	23 January 1996 (23.01.96)	GB
9620759.2	4 October 1996 (04.10.96)	GB

(71) Applicant *(for all designated States except US)*: OXFORD BIOMEDICA (UK) LIMITED [GB/GB]; Medawar Centre, Robert Robinson Avenue, The Oxford Science Park, Oxford OX4 4GA (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: KINGSMAN, Alan, John [GB/GB]; Greystones, Middle Street, Islip, Oxon OX5 2SF (GB). KINGSMAN, Susan, Mary [GB/GB]; Greystones, Middle Street, Islip, Oxon OX5 2SF (GB).

(74) Agent: PRIVETT, Kathryn, Louise; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: RETROVIRAL VECTOR AND ITS USE IN GENE THERAPY

(57) Abstract

DNA sequences for creating replication defective retroviral vector producer cells either *in vivo*, or *in vitro* for reimplantation. The retroviral vector comprises at least one heterologous gene and functional *env* and *gag-pol* genes are absent from the vector so that retrovirus structural proteins are not expressed by the target cells infected and transduced by the vector.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

RETROVIRAL VECTOR AND ITS USE IN GENE THERAPY

This invention relates to DNA sequences encoding retroviral vectors and to sets of DNA sequences encoding retroviral vector particles. The invention also relates to producer cells containing the sequences and capable of producing the retroviral vector and retroviral vector particles containing the vector and to methods of making the producer cells. The invention further relates to uses of the DNA sequences and producer cells in formulations for gene therapy, and to methods of performing gene therapy using the DNA sequences and producer cells.

INTRODUCTION AND PRIOR ART

A number of diseases are amenable to treatment by the delivery of therapeutic nucleic acids to patient's cells. This is referred to as gene therapy (reviewed extensively in Lever and Goodfellow 1995; Culver 1995; Ledley 1995). To achieve gene therapy there must be a method of delivering genes to the patient's cells and additional methods to ensure the effective production of any therapeutic genes. There are two general approaches to achieve gene delivery; these are non-viral delivery and virus-mediated gene delivery. The best characterised virus-mediated gene delivery system uses replication defective retroviruses to stably introduce genes into patients cells. A major disadvantage of non-viral delivery is that the DNA is confined to the initial target cells and is short lived which, for chronic disease, necessitates repeated treatments with the DNA. A major disadvantage of retroviral vectors is that efficient gene transfer is only achieved by transducing cells *ex vivo* and introducing either the transduced cell population back into the patient or grafting in a cell line that is engineered to release retroviral vector particles. These procedures require significant surgical procedure and manipulation of cells. In addition transduction of patients cells with retroviral vector particles is inefficient.

The various known technologies involved in the field of the invention are described in more detail below

1. The production of retroviral vectors from multiple separate DNA sequences

It is known that the separate expression of the components of a retroviral vector on separate DNA sequences cointroduced into the same cell will yield retroviral particles carrying defective retroviral genomes that carry therapeutic genes (e.g. Reviewed by Miller 1992). This cell is referred to as the producer cell. There are two common procedures for generating producer cells. In one, the sequences encoding retroviral Gag, Pol and Env proteins are introduced into the cell and stably integrated into the cell genome; a stable cell line is produced which is referred to as the packaging cell line. The retroviral vector genome is then introduced into the packaging cell line by transfection or transduction to create a stable cell line that has all of the DNA sequences required to produce a retroviral vector particle. The second approach is to introduce the three different DNA sequences that are required to produce a retroviral vector particle i.e. the *env* coding sequences, the *gag-pol* coding sequence and the defective retroviral genome into the cell at the same time by transient transfection and the procedure is referred to as transient triple transfection (Landau & Littman 1992; Pear et al 1993;). The triple transfection procedure procedure has been optimised (Soneoka et al 1995; Finer et al 1994). WO 94/29438 describes the production of producer cells *in vitro* using this multiple DNA transient transfection method and describes the use of these producer cells *in vitro* to transfer retroviral particles to human cells of any lineage that have been removed from a patient. WO 94/19478 describes the use of novel cell lines for producing high titre retroviral stocks following the transient transfection of one or more retroviral plasmids into a packaging cell line. This also describes only the transfer of retroviruses to target cells *in vitro*. The transfer of retroviruses from a producer cell to a target cell *in vitro* is referred to as cocultivation and it is a well established procedure for introducing retroviruses into cells *in vitro*.

2. DNA mediated gene delivery *in vivo*.

The delivery of genes into a variety of different cells in man or animals using naked DNA or DNA associated with a non-viral delivery system has been well described (reviewed by Ledley 1995). The simplest method invoives injecting naked DNA into tissues where it is taken up by a

proportion of cells and the genes contained in the DNA are expressed to produce proteins in these cells (Dubensky et al 1984; Wolffe et al 1990) .

The DNA may be delivered by biolistics; in this procedure metal particles are coated with DNA and projected at high velocity into cells by a high pressure device (e.g. Yang et al 1990). The DNA may be coupled to chemical agents that optimise uptake into cells e.g polylysine or to components of viral particles e.g. adenovirus particles or penton protein or to ligands for specific cognate receptors. The DNA may be encapsulated in liposomes or complexed with cationic lipids (e.g.Hyde et al 1993). Irrespective of how the DNA is delivered by these non-viral methods the seminal feature is that there is no transfer of DNA from the originally transfected cells to other cells except possibly by transfer to daughter cells after cell division. Furthermore the introduced gene is not guaranteed to be permanently maintained in the target cells.

3. Retrovirus mediated gene delivery.

The use of defective retrovirus vectors to deliver genes to target cells is well documented (reviewed by Morgan and Anderson 1993). Defective retroviruses are used to transduce cells that have been removed from the body (*ex vivo* gene delivery) or they can be delivered to tissues *in situ* (*in vivo* gene delivery). The vectors introduce DNA into a cell and it is stably incorporated into the host cell genome where it is expressed to produce any therapeutic gene contained within it. There is no dissemination of the therapeutic gene because retroviral vector mediated gene transfer is a one step event that affects only the initial target cell. *In vivo* gene delivery is not widely used because gene delivery is inefficient largely because the retroviral particles delivered in this way are rapidly cleared from the site of treatment and there is no extended exposure of the cells to viral particles. For example when retroviral vector particles were injected into the brains of rats that were carrying glial tumours only a very few cells were transduced by the vectors due to the short, 2-4hrs, half life of the retroviral particles (Short et al 1990).

4 Implantation of producer cells in target tissues

It has been reported that a producer cell that has been created *in vitro* can be implanted into a tissue *in situ* (Short et al 1990) . The producer cell releases retroviral vector particles which then transduce

neighbouring cells. In this procedure a producer cell is created by the stable transformation of the cell with the DNA sequences specifiying retroviral components *in vitro*. The cell is cultured *in vitro* and then surgically implanted in the patient. The producer cell is foreign and may be short lived due to destruction by the immune system.

Clearly there is a need for improved ways and means for introducing therapeutic genes into patients. Gene therapy would be significantly simplified if stable introduction of DNA into patient cells could be achieved following non-viral DNA delivery and if the effectiveness of non-viral DNA delivery could be improved.

WO 96/17053 describes an adenoviral vector capable of tissue-specific replication due to a regulatory sequence operably linked to the coding region of a gene essential for vector replication. The vector can be used to distribute a polynucleotide in a tissue *in vivo*.

WO 95/22617 describes a retrovirus delivery system in which the vector genome contains a therapeutically active gene in place of the *env* gene, but is otherwise identical to the wild type genome. It is suggested that by introducing an *env* gene into the target cell, retrovirus vector particles may then be produced *in situ*.

THE INVENTION

In one aspect, the invention provides a DNA sequence encoding a replication defective retroviral vector for converting cells in a patient into producer cells capable of producing replication defective retroviral vector particles containing the vector, said retroviral vector comprising at least one heterologous gene, which vector does not contain functional *env* or *gag-pol* genes, the DNA sequence in a form suitable for administering to a patient by non-retroviral means and capable of being taken up by the cells.

The invention also provides a set of DNA sequences for converting cells in a patient into producer cells capable of producing replication defective retroviral vector particles, the set of sequences comprising the DNA sequence above and DNA sequences encoding packaging components Env and Gag-Pol for production of infective retroviral vector particles by the producer cells, the set of DNA sequences in a form suitable for administering to a patient by non-retroviral means and capable of being taken up by the cells.

In another aspect, the invention provides a producer cell capable of producing a replication defective retroviral vector in an infective retroviral vector particle, the producer cell comprising a DNA sequence encoding the replication defective retroviral vector, said vector comprising at least one heterologous gene and which vector does not contain functional *env* or *gag-pol* genes, the producer cell being a fresh cell suitable for introduction into a patient and use in gene therapy.

In a further aspect, the invention provides a method of making a producer cell capable of producing a replication defective retroviral vector said vector comprising at least one therapeutically active gene and which vector does not contain functional *env* or *gag-pol* genes, which method comprises introducing a DNA sequence encoding the replication defective retroviral vector into a fresh mammalian cell to give a producer cell suitable for use in gene therapy.

As will be explained below in more detail, conversion of the fresh mammalian cells to producer cells may be carried out either *in vivo* or *in vitro*. In the *in vitro* case, the producer cells will be suitable for implanting into a patient, and are preferably cells from the patient into whom it is intended to reimplant them.

Further aspects of the invention provide methods of performing gene therapy on a patient, comprising introducing into the patient a producer cell, or a DNA sequence or set of sequences, as described herein; and uses of the producer cells and DNA sequences in formulations for use in gene therapy.

It is particularly preferred that the producer cells according to the various aspects of the invention are of a target cell type for which the therapeutically active gene is intended. This avoids the need to introduce any exogenous cells into the target area in a patient.

However, the producer cells may alternatively be cells capable of delivering the therapeutically active gene to the target cell. Cells of the immune system such as macrophages or tumour infiltrating lymphocytes could be converted to producer cells; this would need to be carried out *in vitro*. When reintroduced into the patent these mobile producer cells will infiltrate organs or tissues and retroviral vector particles from the producer cells will infect those organs or tissues.

The term "fresh mammalian cells" as used here refers to mammalian cells which are in their natural state, or as close as possible to

their natural state. Cells which have been extensively cultured *in vitro*, including cell lines, are not considered to be fresh cells. Fresh cells as referred to herein which have been removed from an organism will usually be primary cells in the sense that they are in a primary culture of cells prepared directly from the tissues of an organism and have not been sub-cultured.

It is an important feature of this invention that the replication defective retroviral vector does not encode any of the structural components of retroviral vector particles. The vector thus allows for the insertion of the therapeutically active gene or genes into the target cell genome, without structural genes encoding Env and Gag-Pol. This avoids problems associated with expression of viral components in the target cells, such as undesired immune responses to those components. For example, cytotoxic T cell responses directed against the products of other foreign genes will be avoided.

Thus a set of DNA sequences according to the invention for producing retroviral vector particles having a replication defective retroviral genome, will comprise:

(i) a DNA construct encoding the vector genome, comprising one or more therapeutically active genes plus the remaining components essential for function such as primer binding site, integration sites, packaging signal.

(ii) a DNA construct or constructs encoding Gag-Pol and Env, preferably as separate constructs.

As will be evident to those skilled in the art, safety considerations to avoid generation of replication competent virus through recombination apply and will be taken into consideration in construction of (i) and (ii) above.

The various DNA constructs described above for use in preparing producer cells according to the invention may be present on separate expression vectors, or they may be present on a single expression vector provided that the vector genome is encoded in a separate transcription unit. The expression vector or vectors will usually be plasmids. Preferably, the DNA constructs encoding the retroviral vector and the required packaging components are administered simultaneously to the patient or delivered simultaneously to the cells being converted to

producer cells *in vitro*. It may therefore be more convenient to incorporate all of the elements onto a single expression vector.

The invention thus comprises the combination of two technologies to produce a method of delivering genes directly to patient/animal tissues such that a long term expression of therapeutic products will be achieved. The essence of the invention is that firstly, using non-viral DNA delivery, combinations of DNA sequences are introduced into the patients cells. The DNA sequence encoding the vector itself, that is the genome of the retrovirus particle, lacks functional *env* and *gag-pol* genes so that the vector is safe and practical. The preferred procedure does not require any removal of cells from the patient. The methods described can however be applied to cells or tissues or organs that are removed from the body and then reimplanted. The combination of DNA sequences is such that when expressed they specify the production of a replication defective retroviral vector genome and the production of the protein components that are required to package that genome to produce retroviral vector particles. The retroviral vector particles are released and then by the process of virus-mediated gene delivery they attach to and enter additional cells and consequently deliver the defective retroviral genome into the cell where it is copied by the particle associated reverse transcriptase and becomes integrated into the genome of those cells. The cell that originally receives the combination of DNA molecules continues to secrete retroviral vector particles for as long as the cell survives or the DNA persists. This creates an extended opportunity for the retroviral vector particles to transduce cells. The retroviral vector particles contain a therapeutic gene which is expressed in the transduced cells. The invention therefore involves establishing a retroviral vector producer cell in the target tissue by the direct delivery of appropriate combinations of DNA sequences.

Details

DNA is delivered to the cells by any appropriate non-retroviral method including injection, biolistic delivery, carrier mediated delivery. Some of the known methods have already been described above. A preferred delivery method for delivering DNA sequences to cells *in vivo* is liposome-mediated delivery. Whichever method is used for *in vivo* delivery, the DNA sequences will need to be provided in a

pharmaceutically acceptable formulation for administering to the patient. Multiple different DNA sequences on separate molecules or a single molecule carrying multiple different sequences are delivered to human/animal cells. These DNA sequences encode the components of a retroviral vector for example the HIT system (Soneoka et al 1995) and the kat system (Finer et al 1994). The DNA sequences encode a retroviral Env protein, a retroviral Gag-Pol protein and a replication defective retroviral genome that is engineered to contain one or more therapeutic genes. Additional sequences may also be included. For example a suicide gene such as HSV Tk might be included on one or all molecules to enable transfected and transduced cells to be destroyed by treatment with drugs such as acyclovir (Plautz et al 1991). The combination of DNA sequences are referred to as the vector production system (VPS). The VPS need not be restricted to the three plasmid systems such as HIT and kat but can comprise any retroviral vector system. Components need not be native retroviral components. For example the *env* gene can be engineered to specify an envelope protein that targets retroviral vector particles to a specific cell type, the vector genome can be engineered to contain gene expression signals that confer special properties on the vector e.g. tissue specific expression, regulated expression and the *gag-pol* gene can be engineered to influence infection and integration for example to deliver DNA into the genome of non-dividing cells or to target DNA to a specific site in the chromosome. The cell that receives the VPS is referred to as an *in situ* retroviral factory (ISRF), it is essentially a retroviral vector producer cell created from one of the patient's own cells. If it is created *in vitro*, using cells removed from the patient, the ISRF will need to be provided in a pharmaceutically acceptable formulation for administering to the patient. The ISRF produces retroviral particles that are released from the cell for as long as the VPS persists, this may be of the order of weeks to months or exceptionally years (Wolff et al 1992). The defective retroviral particles transduce neighbouring cells, referred to as the target cell population (TCP), and deliver the therapeutic gene to those cells as a stably integrated provirus. The TCPs do not produce further virus. The ISRF also expresses the therapeutic gene from the VPS. This combination of non-viral DNA delivery and virus-mediated gene delivery as described allows the dissemination of a therapeutic gene safely, throughout a population of patient's cells.

The invention has a number of advantages which relate to the generation of ISRFs in patients cells both in the body and in tissues removed from the body.

Advantages

i) In the patient

1. Increased efficiency of the delivery of retroviral vectors to target cells in the patient because of the local concentration of viral particles.

2. Increased efficiency of the delivery of retroviral vectors because of the extended time period of exposure of cells to viruses. This means that cells at different stages of the cell cycle have the opportunity to cycle into a phase that is optimum for retroviral infection. These cells would not be available as targets in a single dose treatment with retroviral particles.

3. Creation of an ISRF obviates the need to implant a producer cell that has been generated in the laboratory. Such producer cells are different from patients cells and may even be of non-human origin. These cells are rapidly cleared from most sites of implantation in the body and therefore have limited usefulness.

4. The creation of ISRFs dramatically increases the efficiency of non-viral gene therapy methodologies. In these procedures the transient nature of the expression of the therapeutic gene necessitates frequent multiple repeat treatments with DNA. The ISRF will disseminate therapeutic genes to cells that will continue to produce the product for the life-time of the cell. Treatment need therefore be repeated infrequently if at all for some TCPs.

5. Creation of an ISRF obviates the need to surgically remove patient tissues and transduce them with retroviral vectors before reimplantation. This latter procedure does not allow for further dissemination of the therapeutic gene to other cells. It is technically complex and the cells must be subjected to significant manipulation *in vitro* before reimplantation.

6. When applied to the treatment of tumours, the creation of ISRFs increases the probability of obtaining therapeutic gene expression in

the majority of tumour cells and hence increases the probability of tumour clearance.

7. The ISRF technology has a variety of therapeutic uses, for example but not restricted to:-

i) Cystic fibrosis (CF): The VPS is introduced into lung tissue e.g by liposome mediated DNA delivery. The consequently established ISRFs spread the appropriate therapeutic gene e.g. CFTR (cystic fibrosis transmembrane conductance regulator) throughout the pulmonary tissue. This confers extended relief of the pulmonary symptoms of CF.

ii) Parkinsons disease: The VPS is introduced into cells in the brain by biolistic delivery over a small surgically exposed area. The consequently established ISRFs deliver a retroviral vector to specific cells e.g. glial cells or astrocytes to deliver relevant therapeutic genes e.g. Tyrosine hydroxylase and dopa decarboxylase.

iii) Alzheimers disease: The VPS is introduced into cells in the brain. The consequently established ISRFs deliver the appropriate therapeutic gene e.g. Nerve growth factor.

iv) Tumours: The VPS is delivered to the tumour. The consequently established ISRFs deliver a retroviral vector to surrounding tumour cells to deliver relevant therapeutic genes e.g. HSV thymidine kinase (Tk) or foreign histocompatibility antigens.

ii) In patients tissues *ex vivo*

Advantages i) 1-4 also apply to *ex vivo* applications of ISRFs

1. Direct transduction of a patient's cells *ex vivo* with retroviral vector particles requires the large scale production of high titre retroviral vectors and is often not very efficient requiring prolonged cell culture and genetic selection of transduced cells. An alternative approach is the cocultivation of patients cells with a producer cell line that has previously been created *in vitro*. This may necessitate the separation of the target cells from the producer cell before reimplantation of patients cells. The present invention describes a method to convert a patient's cell directly into a producer cell. The retroviral vector particles are then transferred from the producer cell, now referred to as an ISRF, to other patient cells and the organ/tissue/cells can be reimplanted with minimal manipulation. the

creation of ISRFs therefore obviates the need for cocultivation with non-patient cells or treatment of cells with retroviral vector particles in any *ex vivo* method of gene therapy.

The invention will now be further described in the examples which follow.

EXAMPLES

Example 1.

Construction of an ISRF in a Hela cell monolayer

Hela cells are plated into 60cm dishes and allowed to grow to 80% confluence. Plasmid DNA comprising pHIT456, pHIT111 and pHIT60 is prepared for transfer into Hela cells by standard calcium phosphate precipitation and introduced into cells using the extended overlay method as described in detail in Soneoka et al 1995. pHIT456 contains the amphotropic retroviral envelope gene that allows infection of Hela cells, pHIT111 is a defective retroviral genome that contains the *lac*Z gene and pHIT60 contains the MLV *gag-pol* gene. The coexpression of these plasmids results in the production of retroviral vector particles that can transduce target cells with the *lacZ* gene. This is referred to as configuration A. Briefly, 10μg of each plasmid is coprecipitated with calcium phosphate and the resulting precipitate is placed on the Hela cell monolayer. After 24 hrs the medium is removed and replaced with fresh medium. Replicate dishes are taken at 24hr intervals and the cells are fixed and stained with X-gal to detect the expression of β-galactosidase (Sanes et al 1986). In a control experiment 10μg of plasmid pKV469 is used in place of the retroviral vector plasmid pHIT111. pKV469 is a simple eukaryotic cell expression vector that expresses the *lac*Z gene via the CMV-IE promoter. In this three plasmid configuration no retroviral vector particles are produced This is referred to as configuration B

Cells that are expressing β-galactosidase are stained blue with X-gal. After 24hrs β-galactosidase is expressed in both configurations from the vector plasmid pHIT111 and from pKV469. When the cells are

counted a similar number is observed in each configuration. After 48hrs there is an increase in the number of blue cells in both cases. In configuration B this is due to cell division and adjacent pairs (doublets) of blue cells are observed. In configuration A there is also an increase in the number of cells but this comprises both an increase in doublet cells and an increase in single cells and also the appearance of foci of blue cells. The foci comprise more than two cells which could not result from gene transfer by cell division. The foci appear because virus released from the original cells has infected neighbouring cells. The increase in blue cells in configuration A is more marked after 48 hours with multicellular foci and increased numbers of single cells appearing. This pattern of staining is indicative of one or more rounds of retroviral transduction occurring after the initial transfection of the DNA into the Hela cells. In configuration A, ISRFs are established in the Hela cell monolayer and the *lac*Z gene is disseminated through the target cell population. In configuration B, β-galactosidase expression is resticted to the initially transfected cells and some of their progeny. This experiment establishes that repeated retroviral transduction can occur in a simple homogeneous cell population without the addition of fresh cells as would be the case in a standard cocultivation experiment.

Example 2

Dissemination of the *lac*Z gene throughout the pulmonary tissues of mice

The configuration A and configuration B plasmid sets as above are complexed with cationic liposomes DOTAP or DOTMA/DOPE as described by Alton et al 1993 using 10 to 50μg per plasmid. Liposomes containing DNA are introduced into the lungs of the Edinburgh CF transgenic mouse (Dorin et al 1992) using a jet nebuliser (Alton et al 1993). Mice are sacrificed after 2 days and epithelial cells are harvested by pulmonary lavage. This is repeated for replicate mice at 4 and 14 days. At 14 days lungs are sectioned and sections are stained for the presence of β-galactosidase in pulmonary tissue. In configuration A the number of blue cells increases to a significantly greater extent than in configuration B

and in histological sections foci of blue cells are seen in configuration A but not in configuration B. An ISRF has been established with configuration A and the *lacZ* gene is disseminated through lung tissue.

Example 3

Dissemination of the *lacZ* gene throughout the liver of mice

Mice are subjected to partial hepatectomy. Plasmids in configurations A and B are precipitated by calcium phosphate in the presence of 1μm gold particles. Gold particles are delivered to cells using a biolisitic delivery device. After 2, 4 and 14 days animals are sacrificed and liver sections are stained with X-gal. Foci and scattered blue cells are seen in the liver in configuration A only.

Example 4

Dissemination of the *lacZ* gene throughout the colon of mice

Plasmids in configuration A and B are complexed with cationic liposomes and these are delivered to the colon by instillation. After 2, 4 and 6 days animals are sacrificed and histological sections of the colon are stained with X-gal. Foci and scattered blue cells are seen in colonic epithelium.

Example 5

Dissemination of the *lacZ* gene into the brains of mice.

Plasmids in configuration A and B are introduced in the brains of mice through a surgical window in the cranium. DNA is delivered by a biolistic device. Mice are sacrificed after 4 days and 4 weeks. Foci and scattered blue cells are seen with configuration A only.

Example 6

ISRF using human HT1080 cells in culture:

Three-plasmid co-transfections were carried out by calcium-phosphate precipitation as described in Soneoka *et al.* (1995). Plasmid pHIT 60 (MuLV *gag-pol* expression plasmid), pHIT 456 (amphotropic *env* expression plasmid), and pHIT 111 (proviral DNA construct containing the *lacZ* gene) were co-transfected into HT1080 cells in duplicate sets of five 10cm dishes. In the first instance, these cells were maintained for five days. Every day, one dish from one set was fixed and stained with X-gal, and one dish from the other set was harvested and lysed to measure β-galactosidase activity by a colorimetric assay. As a negative control, HT1080 cells were transfected with pHIT 60, pHIT 123 (ecotropic *env* expression plasmid), and pHIT 111, and the same assays were performed as for the amphotropic producers as described above. Another set of five 10cm dishes was mock-transfected and one dish was harvested everyday to monitor cell growth by counting the number of cells using a hemacytometer. The results obtained are presented in Table 1. The amphotropic virus-producing HT1080 cells showed an increase in *lacZ*-expressing cells and β-galactosidase activity after one day and the levels were maintained thereafter up to day 5. The increase in both *lacZ*-expressing cells and β-galactosidase activity could not be attributed solely to the increase in cell number, since there was no significant cell growth after day 2 and since the ecotropic virus-producing HT1080 cells showed a reduction in the number of *lacZ*-expressing cells (Table 1). In these experiments, polybrene was not used to enhance virus transduction and titers obtained without the use of polybrene at the standard time of 48 hours post-transfection (day 1) were relatively low, approximately 10^3 LFU/ml on NIH 3T3 cells for both ecotropic and amphotropic viruses, and also on HT1080 cells with the amphotropic virus. Therefore, it can be concluded that in one 10cm dish containing 5ml of media, up to 5×10^3 transducible particles were in suspension capable of spreading to surrounding HT1080 cells.

The procedure was repeated but the cells were maintained for 14 days. The transfected cells were passaged on days 5 and 10 at a ratio of 1:5. Again, a similar phenomenon was observed with amphotropic virus-producing HT1080 cells, in that β-galactosidase activity of cells increased after day 1 and was maintained thereafter (Table 2). A significant increase in activity was observed at day 12 (Table 2). This

increase was most likely due to proliferation of cells harboring the *lacZ* gene, rather than the spread of retroviral vectors, as no infectious virus was being produced at this stage (Table 2). β-galactosidase activity of ecotropic virus-producing HT1080 cells remained at almost baseline level throughout the 14 days, suggesting that the *lacZ* gene transduced by the amphotropic virus was maintained and stably expressed.

No significant cell growth appeared to occur during the course of either experiment, except only after the first day and probably after passage of the cells on days 5 and 10 in the second experiment (Tables 1 and 2). Cells were transfected at approximately 80% confluency, since some toxicity appears to occur from the calcium-phosphate transfection. Recovery of cells from the transfection may explain the growth of cells between days 1 and 2. By day 2, the cells were nearing confluency, hence, the lack of detectable cell growth. Although the nature of MuLV requires that cells be in an actively-dividing state for infection to occur, the low virus titers produced from day 3 onwards (Table 2) suggests that growth of the cells after day 2 was insignificant.

Taken together these data demonstrate that it is possible to use in situ retroviral factories as effective means of spreading useful retroviral vectors through a population of cells.

Table 1 Spread of retroviral vector in HT1080 retroviral producer cells maintained for 5 days

TYPE OF VIRUS PRODUCED[a]	DAY[b]	# OF BLUE CELLS[c]	β-GAL ACTIVITY[d]	# OF CELLS[e]
Amphotropic (pHIT60, pHIT456, pHIT111)	1	4	0.02	N/A[f]
	2	10.4	0.25	N/A
	3	10.4	0.22	N/A
	4	11.2	0.22	N/A
	5	10.1	0.24	N/A
Ecotropic (pHIT60, pHIT123 pHIT111)	1	4.5	0.02	N/A
	2	1.6	0	N/A
	3	1	0	N/A
	4	0.7	0	N/A
	5	0.6	0	N/A
Mock transfected	1	N/A	N/A	3.4×10^6
	2	N/A	N/A	7.3×10^6
	3	N/A	N/A	4.0×10^6
	4	N/A	N/A	5.7×10^6
	5	N/A	N/A	7.0×10^6

[a] Three-plasmid co-transfections were performed as described.

[b] Day 1 corresponds to 48 hours post-transfection.

[c] Cells were fixed and stained with X-gal. The number of blue cells was counted under x40 magnification and the average of 10 fields was recorded.

[d] 100μg of total protein extract was used and absorbance was measured at OD_{420}.

[e] Total number of cells in a 10cm dish was determined using a hemacytometer.

[f] Not applicable.

Table 2 Spread of retroviral vector in HT1080 retroviral producer cells maintained for 14 days

VIRUS TYPE[a]	DAY[b]	β-GAL ACTIVITY[c]	TITERS[d] NIH 3T3	# OF HT1080	CELLS[e]
Amphotropic	1	0.023	3.4×10^3	3×10^2	N/A[f]
	2	0.036	ND[g]	ND	N/A
	3	0.094	3.8×10^2	53	N/A
	4	0.061	ND	ND	N/A
	5	ND	1	0	N/A
	8	0.053	0	0	N/A
	12	0.355	0	0	N/A
	14	0.310	0	0	N/A
Ecotropic	1	0.014	1.8×10^3	0	N/A
	2	0.033	ND	ND	N/A
	3	0.018	3.8×10^2	0	N/A
	4	0.026	ND	ND	N/A
	5	ND	0	0	N/A
	8	0.008	0	0	N/A
	12	0.014	0	0	N/A
	14	0.074	0	0	N/A
Mock-transfected	1	N/A	N/A		2.7×10^6
	2	N/A	N/A		7.2×10^6
	3	N/A	N/A		7.5×10^6
	4	N/A	N/A		7.4×10^6
	8	N/A	N/A		8.7×10^6
	12	N/A	N/A		4.4×10^6
	14	N/A	N/A		6.2×10^6

a Three-plasmid co-transfections were performed as described . Amphotropic: pHIT60, pHIT456, pHIT111; Ecotropic: pHIT60, pHIT123, pHIT111.

b Day 1 corresponds to 48 hours post-transfection. Cells were split at a ratio of 1:5 on days 5 and 10.

c 30μg of total protein extract was used and absorbance was measured at OD_{420}.

d Titers were obtained by harvesting supernatant from each 10cm dish, filtering through 0.45μm filters, and adding viral supernatant to either NIH 3T3 cells or HT1080 cells in the absence of polybrene. Cells were X-gal stained 48 hours later and titers were obtained in *lacZ*-forming units per ml (LFU/ml)

e Total number of cells from mock-transfected 10cm dishes was counted using a hemacytometer

f Not applicable.

g Not done.

REFERENCES

Alton, E. et al 1993 Nature Genet. 5, 135
Dorin, J.R. et al 1992. Trans. Res. 1, 101.
Dubensky, T.W. 1984. Proc. Natl. Acad. Sci. 81, 7529
Finer M. et al. 1994. Blood, 83, 43.
Hyde, S.C 1993, Nature 362, 250
Landau, N.R. & Littman, D. 1992. J. Virol. 66, 5110
Ledley, F.D. 1995. Hum. Gene Ther. 6, 1129.
Lever and Goodfellow 1995; Br. Med Bull., 51, 1-242;
Miller, A.D. 1992. Curr. Topics in Microbiol and Immunol. 158, 1-24
Morgan and Anderson. 1993. Annual Review of Biochem., 62, 192
Pear W.S. et al 1993. Proc. Natl. Acad. Sci. 90, 8392
Plautz G. et al. 1991 New Biol.7, 709
Sanes, J.R., et al . 1986 EMBO J., 5 3133.
Short M.P.et al 1990. J. Neuroscience Res. 27, 427
Soneoka Y. et al. 1995. Nucl. Acids Res. 23, 628
Wolff, J.A. 1990. Science, 247, 1465.
Yang, N.S. et al 1990, Proc. Natl. Acad. Sci, 87, 9568.

CLAIMS:

1. A DNA sequence encoding a replication defective retroviral vector for converting cells in a patient into producer cells capable of producing replication defective retroviral vector particles containing the vector, said retroviral vector comprising at least one heterologous gene, which vector does not contain functional *env* or *gag-pol* genes, the DNA sequence in a form suitable for administering to a patient by non-retroviral means and capable of being taken up by the cells.

2. A set of DNA sequences for converting cells in a patient into producer cells capable of producing replication defective retroviral vector particles, the set of sequences comprising the DNA sequence according to claim 1 and DNA sequences encoding packaging components Env and Gag-Pol for production of infective retroviral vector particles by the producer cells, the set of DNA sequences in a form suitable for administering to a patient by non-retroviral means and capable of being taken up by the cells.

3. DNA sequences according to claim 1 or claim 2, wherein the at least one heterologous gene includes at least one therapeutically active gene.

4. DNA sequences according to any one of claims 1 to 3, for converting cells of the patient which are of a target cell type intended for receiving the therapeutically active gene.

5. DNA sequences according to any one of claims 1 to 4, present in one or more plasmids.

6. A producer cell capable of producing a replication defective retroviral vector in an infective retroviral vector particle, the producer cell comprising a DNA sequence encoding the replication defective retroviral vector, said vector comprising at least one heterologous gene and which vector does not contain functional *env* or *gag-pol* genes, the producer cell being a fresh cell suitable for introduction into a patient and use in gene therapy.

7. The producer cell according to claim 6, wherein the at least one heterologous gene in the vector includes at least one therapeutically active gene.

8. The producer cell according to claim 7, wherein the cell is of a target cell type intended for receiving the therapeutically active gene.

9. The producer cell according to claim 7, wherein the cell is an immune system cell capable of delivering the vector to target cells intended to receive the therapeutically active gene.

10. The producer cell according to any one of claims 6 to 9, for reimplantation into the patient from which it is derived.

11. A method of making a producer cell capable of producing a replication defective retroviral vector said vector comprising at least one therapeutically active gene and which vector does not contain functional *env* or *gag-pol* genes, which method comprises introducing a DNA sequence encoding the replication defective retroviral vector into a fresh mammalian cell to give a producer cell suitable for use in gene therapy.

12. The method according to claim 11, wherein a set of DNA sequences comprising the DNA sequence of claim 11 and DNA sequences encoding packaging components Env and Gag-Pol, are introduced into the cell.

13. The method according to claim 11 or claim 12, wherein the producer cell is of a target cell type intended for receiving the therapeutically active gene.

14. The method according to claim 11 or claim 12, wherein the producer cell is an immune system cell capable of delivering the vector to target cells intended to receive the therapeutically active gene.

15. The method according to any one of claims 11 to 13, wherein the fresh cell is a patient's cell and is converted to a producer cell *in vivo* in the patient.

16. The method according to any one of claims 11 to 14, wherein the DNA sequence or set of DNA sequences is introduced into the fresh cell *in vitro*.

17. The method according to claim 16, wherein the fresh cell is from a patient and the resulting producer cell is subsequently reimplanted into the patient.

18. A method of performing gene therapy on a patient, which method comprises introducing into the patient a producer cell according to any one of claims 6 to 10.

19. A method of performing gene therapy on a patient, which method comprises introducing into the patient by non-retroviral means a DNA sequence or set of sequences according to any one of claims 1 to 5.

20. Use of a producer cell according to any one of claims 6 to 10, in a formulation for use in gene therapy.

21. Use of a DNA sequence or set of DNA sequences according to any one of claims 1 to 5, in a formulation for use in gene therapy.

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/GB 97/00210

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N15/87 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 22617 A (UNIV PARIS CURIE ;KLATZMANN DAVID (FR); SALZMANN JEAN LOUP (FR)) 24 August 1995 cited in the application see the whole document ---	1-21
A	WO 94 29438 A (CELL GENESYS INC) 22 December 1994 cited in the application see the whole document ---	1-21
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 April 1997	14.05.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Olsen, L

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr ial Application No
PCT/GB 97/00210

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NUCLEIC ACIDS RESEARCH, vol. 23, no. 4, 25 February 1995, pages 628-633, XP000569533 SONEOKA Y ET AL: "A TRANSIENT THREE-PLASMID EXPRESSION SYSTEM FOR THE PRODUCTION OF HIGH TITER RETROVIRAL VECTORS" cited in the application see the whole document	1-21
A	HEMATOLOGY/ONCOLOGY CLINICS OF NORTH AMERICA, vol. 9, 1995, pages 91-113, XP000653407 LEE, J H AND KLEIN, H G : "CELLULAR GENE THERAPY" see the whole document	1-21
A	CURRENT OPINION IN GENETICS AND DEVELOPMENT, vol. 3, 1993, pages 110-114, XP000653497 GROSSMAN, M AND WILSON, J M: "RETROVIRUSES: DELIVERY VEHICLE TO THE LIVER" see the whole document	1-21

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 97/00210

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9522617 A	24-08-95	FR 2716461 A	25-08-95
		FR 2716459 A	25-08-95
		AU 1851895 A	04-09-95
		CA 2183151 A	24-08-95
		EP 0738327 A	23-10-96
		FI 963246 A	16-10-96
		NO 963358 A	02-09-96
		ZA 9501451 A	18-12-95
WO 9429438 A	22-12-94	AU 7246294 A	03-01-95
		EP 0710280 A	08-05-96
		JP 8511169 T	26-11-96

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB 97/00210

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N15/87 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 22617 A (UNIV PARIS CURIE ;KLATZMANN DAVID (FR); SALZMANN JEAN LOUP (FR)) 24 August 1995 cited in the application see the whole document ---	1-21
A	WO 94 29438 A (CELL GENESYS INC) 22 December 1994 cited in the application see the whole document --- -/--	1-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

7. 05. 97

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer
Olsen, L

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NUCLEIC ACIDS RESEARCH, vol. 23, no. 4, 25 February 1995, pages 628-633, XP000569533 SONEOKA Y ET AL: "A TRANSIENT THREE-PLASMID EXPRESSION SYSTEM FOR THE PRODUCTION OF HIGH TITER RETROVIRAL VECTORS" cited in the application see the whole document	1-21
A	HEMATOLOGY/ONCOLOGY CLINICS OF NORTH AMERICA, vol. 9, 1995, pages 91-113, XP000653407 LEE, J H AND KLEIN, H G : "CELLULAR GENE THERAPY" see the whole document	1-21
A	CURRENT OPINION IN GENETICS AND DEVELOPMENT, vol. 3, 1993, pages 110-114, XP000653497 GROSSMAN, M AND WILSON, J M: "RETROVIRUSES: DELIVERY VEHICLE TO THE LIVER" see the whole document	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/00210

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9522617 A	24-08-95	FR 2716461 A	25-08-95
		FR 2716459 A	25-08-95
		AU 1851895 A	04-09-95
		CA 2183151 A	24-08-95
		EP 0738327 A	23-10-96
		FI 963246 A	16-10-96
		NO 963358 A	02-09-96
		ZA 9501451 A	18-12-95
WO 9429438 A	22-12-94	AU 7246294 A	03-01-95
		EP 0710280 A	08-05-96
		JP 8511169 T	26-11-96